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The consensus motif for phosphorylation by cyclin D1–Cdk4 is different from that for phosphorylation by cyclin A/E–Cdk2

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Cyclin D–Cdk4/6 and cyclin A/E–Cdk2 are suggested to be involved in phosphorylation of the retinoblastoma protein (pRB) during the G_1/S transition of the cell cycle. However, it is unclear why several Cdks are needed and how they are different from one another. We found that the consensus amino acid sequence for phosphorylation by cyclin D1–Cdk4 is different from S/T-P-X-K/R, which is the consensus sequence for phosphorylation by cyclin A/E–Cdk2 using various synthetic peptides as substrates. Cyclin D1–Cdk4 efficiently phosphorylated the G1 peptide, RPPTLS^{780}PIPHIPR that contained a part of the sequence of pRB, while cyclins E–Cdk2 and A–Cdk2 did not. To determine the phosphorylation state of pRB *in vitro* and *in vivo*, we raised the specific antibody against phospho-Ser780 in pRB. We confirmed that cyclin D1–Cdk4, but not cyclin E–Cdk2, phosphorylated Ser780 in recombinant pRB. The Ser780 in pRB was phosphorylated in the G_1 phase in a cell cycle-dependent manner. Furthermore, we found that pRB phosphorylated at Ser780 cannot bind to E2F-1 *in vivo*. Our data show that cyclin D1–Cdk4 and cyclin A/E Cdk2 phosphorylate different sites of pRB *in vivo*.

Keywords: Cdk2/Cdk4/cell cycle/cyclin D1/p107/RB protein

Introduction

Cyclin-dependent kinases (Cdks) play important roles in the regulation of the cell cycle (Pines, 1993; Sherr, 1995). Several genes encoding Cdks [such as *cdc2(cdk1)*, *cdk2*, *cdk3*, *cdk4*, *cdk5*, *cdk6* and *cdk7*] have been cloned. Cdk2 associates with cyclin E and cyclin A (Pines, 1993) and the resultant kinase activities increase at the G_1/S boundary and in early S phase respectively (Rosenblatt *et al.*, 1992; Pagano *et al.*, 1993; Ohtsubo *et al.*, 1995). D-type cyclins, which are activated by rearrangement or amplification in several tumors, associate with Cdk4 and Cdk6 (Sherr, 1995). Recently, it was suggested that proteins that inhibit Cdk4/6 (namely p16/MTS1 and p15/MTS2) regulate the cell cycle progression through G_1 phase and that their genes could be candidates for tumor-suppressor genes (Kamb, 1995). The D-type cyclin–Cdk4 complexes phosphorylate the retinoblastoma protein (pRB, a product of one of the tumor suppressor genes) and regulate the cell cycle during G_1/S transition (Taya, 1995; Weinberg, 1995). Direct binding between pRB and D-type cyclins is important for the accessibility of D-type cyclin kinase to pRB (Kato *et al.*, 1993). The cyclin D1-dependent kinases seem to be involved in G_1/S progression since microinjection of antibodies against cyclin D1 inhibits entry into the S phase (Baldin *et al.*, 1993; Lukas *et al.*, 1995a; Roussel *et al.*, 1995). The growth-suppressive activity of pRB is mainly exerted through its binding to transcription factors such as the E2F family, which regulate expression of several growth-promoting genes (Helin and Harlow, 1993). The complexes of E2Fs bound to pRB are dissociated upon phosphorylation of pRB by RB kinase at G_1/S (Dynlacht *et al.*, 1994; Takahashi *et al.*, 1995). Cyclin D–Cdk4, cyclin E–Cdk2 and cyclin A–Cdk2 phosphorylate pRB and are candidates for RB kinases *in vivo*. The site of phosphorylation of pRB has been studied (Taya *et al.*, 1989, 1990; Lees *et al.*, 1991; Hamel *et al.*, 1992; Mittnacht *et al.*, 1994), but differences in the sites of phosphorylation by Cdk2 and Cdk4 have not been clarified. It is also unclear about functional differences between cyclin D–Cdk4 and cyclin E–Cdk2 in the progression of G_1 phase. Cyclin D–Cdk4 efficiently phosphorylates pRB but not H1 histone, whereas Cdk2 phosphorylates both proteins (Matsushime *et al.*, 1992, 1994; Ewen *et al.*, 1993; Kato *et al.*, 1993). From the evidence, it is postulated that Cdk4 and Cdk2 may recognize different amino acid sequences. We studied the substrate specificities using purified cyclin D1–Cdk4, cyclin A–Cdk2 and cyclin E–Cdk2. In this report, we show that the consensus sequence for phosphorylation by cyclin D1–Cdk4 is different from that for phosphorylation by cyclin A/E–Cdk2.

Positions of the amino acids in synthetic peptides are abbreviated. Serine and threonine residues that are expected to be sites of phosphorylation by cyclin-dependent kinases are designated Ser^0 and Thr^0 respectively. For example, the threonine residue at the third position before Ser^0 of the G1 peptide (RPPTLS^0 PIPHIPR) is designated T^{-2} or Thr^{-2} and the histidine residue at the fifth position after Ser^0 of the G1 peptide (RPPTLS^0 PIPHIPR) is designated H^{+4} or His^{+4}.

Recombinant baculoviruses that encoded cDNAs for proteins such as cyclin D1 and Cdk4 are abbreviated as cyclin D1-virus and Cdk4-virus, respectively.

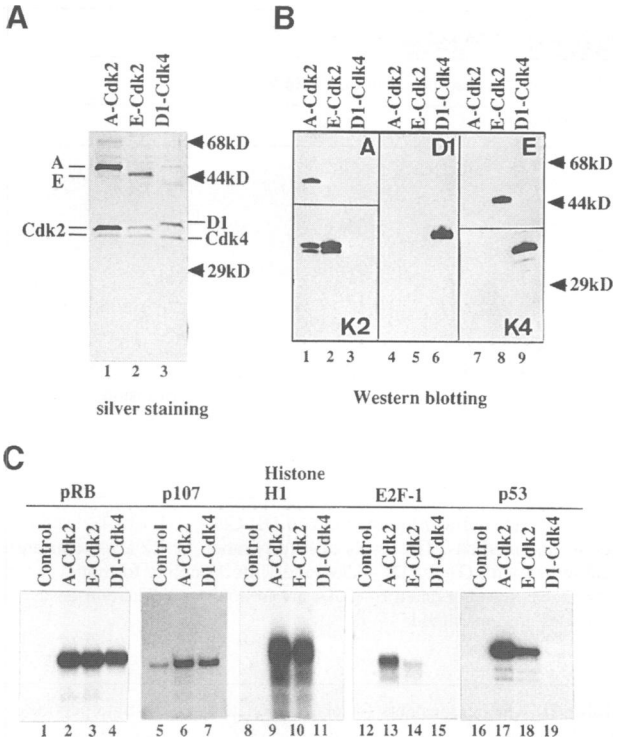

Fig. 1. Characterization of purified cyclin–Cdk complexes. Cyclin A–Cdk2 (A-Cdk2), cyclin E–Cdk2 (E-Cdk2) and cyclin D1–Cdk4 (D1-Cdk4) were extensively purified from Sf9 cells that had been co-infected with baculoviruses that encoded each cyclin and Cdk as described in Materials and methods. These cyclin–Cdks were subjected to SDS–PAGE (12.5% polyacrylamide) and then to silver staining (**A**) and Western blotting (**B**) with antibodies against cyclin A (A), cyclin E (E), cyclin D1 (D1), Cdk2 (K2) and Cdk4 (K4). (**C**) Phosphorylation of proteins by purified cyclin D1–Cdk4 *in vitro*. Purified cyclin A–Cdk2 (A-Cdk2), cyclin E–Cdk2 (E-Cdk2) and cyclin D1–Cdk4 (D1-Cdk4) were incubated with various proteins in R buffer that contained [γ-^{32}P]ATP at 30°C for 30 min. pRB, GST–E2F-1, H1 histone and p53 were purified proteins and p107 was prepared by immunoprecipitation with p107-specific antibody, as described in Materials and methods. Phosphorylated proteins in the reaction mixtures were separated by SDS–PAGE (7.5% polyacrylamide) and detected by autoradiography.

Results

Characterization of purified cyclin–Cdk

Cyclin D1–Cdk4 phosphorylates pRB but scarcely phosphorylates H1 histone, whereas cyclin A/E–Cdk2 phosphorylates both pRB and H1 histone, as reported previously (Matsushime *et al.*, 1992, 1994; Ewen *et al.*, 1993; Kato *et al.*, 1993). To investigate the differences in substrate specificity between Cdk4 and Cdk2, we examined the phosphorylation of various synthetic peptides that contained sequences from either pRB or H1 histone by purified cyclin D1–Cdk4 and by cyclin A/E–Cdk2. Active cyclin D1–Cdk4, cyclin A–Cdk2 and cyclin E–Cdk2 were produced by baculovirus expression systems, as described previously (Kitagawa *et al.*, 1995). These Cdk–cyclin complexes were highly purified by HPLC, as described in Materials and methods. As shown in Figure 1, levels of both cyclin D1 and Cdk4 were equivalent in terms of molarity in the final preparation of cyclin D1–Cdk4, which was highly purified and contained no cyclin A/E or Cdk2 protein (Figure 1A, lane 3 and B lanes 3, 6 and 9). Figure 1C shows the results of phosphorylation of various proteins by Cdks. pRB, p107, H1 histone and p53 (containing the Ser/Thr-Pro-X-basic motif) were phosphorylated by both cyclin A–Cdk2 and cyclin E–Cdk2. We confirmed that the purified cyclin D1–Cdk4 phosphorylated pRB but not H1 histone, whereas cyclin A–Cdk2 and also cyclin E–Cdk2 efficiently phosphorylated both pRB and H1 histone (Figure 1C, lanes 1–4 and 8–11 respectively). E2F-1 was phosphorylated by cyclin A–Cdk2 rather than by cyclin E–Cdk2, as a consequence of the direct binding of cyclin A to E2F-1 (Krek *et al.*, 1994; Kitagawa *et al.*, 1995). p107 was phosphorylated by cyclin D1–Cdk4 (Figure 1C, lane 7), as was pRB (lane 4). However, E2F-1, p53 and H1 histone were not phosphorylated by cyclin D1–Cdk4 (Figure 1C, lanes 11, 15 and 19). Wang and Prives (1995) reported that p53 is a good substrate for cyclin B–Cdc2 and cyclin A–Cdk2 but a poor substrate for cyclin E–Cdk2 and cyclin D1–Cdk4. Because p53 contains S-P-X-P-K which is located in PNNTSSSPQPKKKKP, one of the consensus motifs for phosphorylation by Cdk2/Cdc2 but not by cyclin D1–Cdk4, cyclin D1–Cdk4 may not phosphorylate p53. However, it is still not known why cyclin E–Cdk2 could not efficiently phosphorylate p53.

Cdk4 and Cdk2 recognize different amino acid sequences

We previously defined the sites of phosphorylation of pRB using synthetic peptides that contained part of the sequence of pRB (Taya *et al.*, 1989). Similarly, in this study, we investigated the substrate specificity of the purified cyclin–Cdk complexes using various synthetic peptides. It has been reported that more than 10 sites of pRB are phosphorylated *in vivo* (Lees *et al.*, 1991). We postulated that Cdk4 and Cdk2 might phosphorylate different sites in pRB. First, several synthetic peptides containing a part of the sequence of pRB were tested for phosphorylation by cyclin D1–Cdk4, cyclin A–Cdk2 and cyclin E–Cdk2. Rates of phosphorylation of these peptides are shown in Table I. Interestingly, the G1 peptide (RPPTLSPIPHIPR) was found to be the best substrate for cyclin D1–Cdk4 although it was a very poor substrate for both cyclin A–Cdk2 and cyclin E–Cdk2. The B peptide (INGSPRTPR-RGQNR) and H peptide (KFPSSPLRIPGGNI), which have proline residues at positions –2 or –3 relative to the putative sites of phosphorylation, were good substrates for cyclin D1–Cdk4. These results suggested that proline residues at position –2 or –3 might be important for recognition by cyclin D1–Cdk4.

Consensus motif for phosphorylation by cyclin D1–Cdk4

From the results described above, we expected that the S1 peptide of histone H1 might be a good substrate for Cdk4 if single amino acids were replaced. Therefore, we synthesized many peptides that contained sequences derived from the S1 and G1 peptides to determine the consensus sequence for phosphorylation by cyclin D1-Cdk4. Kinetic constants for some of the typical peptides for reactions catalyzed by cyclin D1–Cdk4 were determined using Lineweaver–Burk plots (Table II). The larger the value of V_{max}/K_m, the better was a peptide as a substrate for cyclin D1–Cdk4. The S4 peptide, as well as the S11 and S13 peptides (which included Pro^{-2}), gained a larger value of V_{max}/K_m, so they were good substrates for Cdk4.

Table I. Phosphorylation of synthetic peptides that contained sequences found in pRB by cyclin D1–Cdk4

Peptide		Levels of incorporation of ^{32}P in 30 min		
		D1–Cdk4	A–Cdk2	E–Cdk2
pRB				
A	$_{1}$MPPK**T**PRKTAATAA	7.8	47	75
B	$_{246}$INGSPR**T**PRRGQNR	43	100	100
C	$_{351}$FETQR**T**PRKSNLDE	1.9	7.2	6.2
D2	$_{603}$ADMYL**S**PVR**S**PK	8.3	11	11
F	$_{806}$I**S**PLK**S**PYKISEG	3.7	19	41
G1	$_{775}$RPPTL**S**PIPHIPR	100	1.6	3.8
H	$_{791}$KFPS**S**PLRIPGGNI	35	11	37
I	$_{802}$GNIYI**S**PLK**S**PYKI	8.0	52	47
J	$_{818}$GLP**T**PT**K**MTPRSRIL	9.2	33	not tested
Histone H1				
S1	AKAKK**T**PKKAKK	1.7	155	105

Synthetic peptides that contained sequences from pRB (A–J) or histone H1 (S1) were incubated with purified cyclin D1–Cdk4 (D1–Cdk4), cyclin A–Cdk2 (A–Cdk2) or cyclin E–Cdk2 (E-Cdk2) at 200 μM as described in Materials and methods. The levels of incorporation of ^{32}P are the means of results of triplicate assays in 30 min and are normalized to the incorporation observed with G1 for D1–Cdk4, and with B peptide for both A–Cdk2 and E–Cdk2 which was assumed to be 100%. Predictable Ser and Thr residues phosphorylated by Cdk4 are in bold type. The consensus motifs for phosphorylation by Cdk2/Cdc2, Ser or Thr-Pro-X-Lys/Arg, in these peptides are underlined.

In the presence of Pro^{-2}, Leu^{-1} increased the capacity of the peptide to serve as a substrate for Cdk4, as compared with Ala^{-1}. The presence of basic amino acids (such as Lys^{+2}-Lys^{+3}) was necessary. Relative capacities of the other S-series peptides as substrates for cyclin D1–Cdk4 as well as for both cyclin E–Cdk2 and MAP kinase are shown in Table III. Cyclin A–Cdk2 and cyclin E–Cdk2 recognized the same consensus sequence (S/T-P-X-K/R) for phosphorylation, but their kinetic constants against several S series peptides were different (Higashi *et al.*, 1995). As expected, introduction of a proline residue at position –2 increased the capacity to serve as a substrate for cyclin D1–Cdk4. The presence of Pro^{+1} was critical for phosphorylation by Cdk4, as well as by Cdk2, because the S5 peptide (with alanine at this position) was scarcely phosphorylated by Cdk4. Ser^{0} instead of Thr^{0} increased the capacity of the peptide to serve as a substrate for Cdk4 (see S11 and S11S in Tables II and III). Lys^{+3}/Arg^{+3} also seemed important in the presence of Pro^{-2} (see S4, S6, S7, S10 and S14). Leu^{-1} increased the capacity of the peptide to serve as a substrate for Cdk4, as compared with Ala^{-1} or Lys^{-1} (see S3, S4 and S11). These data suggested that Pro^{-2}-Leu^{-1}-Ser^{0}/Thr^{0}-Pro^{+1}-Lys^{+2}-Arg^{+3}/Lys^{+3}, represented by S11S/S11, might be a suitable sequence for a substrate for Cdk4. Cdk2 and MAP kinase also phosphorylated these peptides. However, Pro^{-2} was unnecessary for substrates for Cdk2 (see S1, S12, S13, S14 and S15). MAP kinase required Pro^{-2} but not Lys^{+2}-Arg^{+3}/Lys^{+3} (see S11, S14 and S15).

The G1 peptide (RPPTLSPIPHIPR) was phosphorylated by Cdk4 but not by Cdk2, as described above. We further studied the importance of each residue in the G1 peptide using various derivatives of this peptide (Tables II and IV). Substitution of Ile^{+2}-Pro^{+3}-His^{+4} by Ala^{+2}-Ala^{+3}-Ala^{+4} caused a remarkable decrease in the capacity to serve as a substrate for Cdk4 (see G1 and G13 in Table IV). By contrast, Ile^{+2}-Pro^{+3}-His^{+4} was not a good sequence for a substrate for Cdk2 because replacement of Ile^{+2}-Pro^{+3}-His^{+4} by Lys^{+1}-Lys^{+2}-Ala^{+3} restored the capacity to serve as a substrate for Cdk2. Substitution of

Table II. Kinetic constants for phosphorylation of substrate peptides by D1–Cdk4

Peptide	Sequence	K_m (mM)	V_{max} (pmol/min)	V_{max}/K_m
S1	AKAKK**T**PKKAKK	0.180	0.0185	0.103
S4	KAPA**T**PKKAK	1.16	0.907	0.782
S11	KAPL**T**PKKAK	0.442	0.877	1.98
S11S	KAPL**S**PKKAK	0.403	1.62	4.02
S13	KAAL**T**PKKAK	1.21	0.102	0.0843
S14	KAPL**T**PAAKK	2.20	0.434	0.197
G1	RPPTL**S**PIPHIPR	2.57	2.24	0.872
G11	RAAPL**S**PIPHIPR	1.40	2.42	1.73
G12	RPPTA**S**PIPHIPR	0.551	0.128	0.232

Some typical S series peptides derived from the S1 peptide as well as three G series peptides based on the sequence of the G1 peptide were incubated with purified cyclin D1–Cdk4 as described in Materials and methods. Kinetic constants for them were determined using Lineweaver–Burk plots. The larger was the value of V_{max}/K_m, the better was a peptide as a substrate for cyclin D1–Cdk4. Predictable Ser and Thr residues phosphorylated by Cdk4 are in bold type.

Leu^{-1} by Lys^{-1} or Ala^{-1} decreased the capacity to act as a substrate for Cdk4 (G1, G9 and G12). MAP kinase also phosphorylated the G series peptides. However, the G4 peptide was a relatively better substrate for Cdk4 than for MAP kinase. The G11 peptide, containing Pro^{-2}, was 250% more effective for Cdk4 than the G1 peptide.

These data suggested that the most powerful motif for recognition and phosphorylation by Cdk4 is Pro^{-2}-Leu^{-1}-Ser^{0}/Thr^{0}-Pro^{+1}-X^{+2}-Lys^{+3}/Arg^{+3}/His^{+3} or Pro^{-2}-Leu^{-1}-Ser^{0}/Thr^{0}-Pro^{+1}-Ile^{+2}-Pro^{+3}-Lys^{+4}/Arg^{+4}/His^{+4}, among the possibilities that we examined.

Cyclin D1-dependency of Cdk4 kinase activities

As described above, purified cyclin D1–Cdk4 phosphorylated S4, S11, G1 and G10 but not S1. We attempted to confirm this property of cyclin D1–Cdk4 using lysates prepared from Sf9 cells that had been infected with both cyclin D1- and Cdk4-virus. Lysates prepared from Sf9 cells co-infected with cyclin D1-virus and Cdk4-virus

Table III. Phosphorylation of the S series of peptides by Cdk4, Cdk2 and MAP kinase

Peptide		Levels of incorporation of ^{32}P in 30 min		
		D1–Cdk4	E–Cdk2	MAP kinase
S1	AKAKK**T**PKKAKK	2.5	41	0.32
S2	AKAKK**T**PKAKKK	0.28	0.019	0.78
S3	KAPK**T**PKKAK	29	95	37
S4	KAPA**T**PKKAK	38	98	43
S5	KAPA**T**AKKAK	0.038	0.00	3.3
S6	KAPA**T**PKAKK	17	21	41
S7	KAPA**T**PAAKK	5.8	67	30
S8	KPAA**T**PKKAK	2.8	70	4.8
S9	KPPA**T**PKKAK	29	100	100
S10	KAPA**T**PKRAK	85	30	31
S11	KAPL**T**PKKAK	100	96	78
S11S	KAPL**S**PKKAK	203	not tested	not tested
S12	KAAP**T**PKKAK	2.4	71	3.4
S13	KAAL**T**PKKAK	17	73	10
S14	KAPL**T**PAAKK	5.0	4.8	74
S15	KAAL**T**PAAKK	0.85	7.7	2.6

The S series of peptides was incubated with purified cyclin D1–Cdk4 (D1–Cdk4), cyclin E–Cdk2 (E–Cdk2) and MAP kinase at 200 μM as described in Materials and methods. The levels of incorporation of ^{32}P are the means of results of triplicate assays in 30 min and are normalized to the incorporation observed with S11 for D1–Cdk4, and with S9 for both E-Cdk2 and MAP kinase which was assumed to be 100%.

Table IV. Phosphorylation of the G series of peptides by Cdk4, Cdk2 and MAP kinase

Peptide		Levels of incorporation of ^{32}P in 30 min		
		D1–Cdk4	E–Cdk2	MAP kinase
G1	RPPTL**S**PIPHIPR	38	0.0	9.0
G2	RPPTL**S**PIPKIPR	89	5.1	18
G3	RPPTL**S**PIPAIPR	20	0.0	3.6
G4	RPPTL**S**PIAHIPR	26	0.0	3.6
G5	RPPTL**S**PKKAKR	21	100	4.8
G6	RPATL**S**PIPHIPR	34	0.0	7.1
G7	RAPTL**S**PIPHIPR	46	0.0	9.9
G8	RAATL**S**PIPHIPR	38	0.0	8.5
G9	RPPTK**S**PIPHIPR	14	0.0	2.7
G10	RAPPL**S**PIPHIPR	51	0.37	100
G11	RAAPL**S**PIPHIPR	100	0.0	74
G11T	RAAPL**T**PIPHIPR	49	0.0	not tested
G12	RPPTA**S**PIPHIPR	6.2	5.7	2.8
G13	RPPTL**S**PAAAKR	6.4	2.1	1.6

The G series of peptides was also phosphorylated as described in Table III. The levels of incorporation of ^{32}P are the means of results of triplicate assays in 30 min and are normalized to the incorporation observed with G11 for D1–Cdk4, with G5 for E–Cdk2 and with G10 for MAP kinase, which was assumed to be 100%.

phosphorylated the S4, S11, G1 and G10 peptides but not the S1 peptide in a dose-dependent manner (Figure 2). pRB was also phosphorylated in a similar manner by the crude lysates (data not shown). Lysates prepared from Sf9 cells infected with wild-type, cyclin D1- or Cdk4-virus alone did not significantly phosphorylate any of these peptides (Figure 2). The S1 peptide was, however, phosphorylated non-specifically by all lysates. These data were consistent with those obtained with purified cyclin D1–Cdk4 and suggested that phosphorylation of the S4, S11, G1 and G10 peptides are dependent on the action of cyclin D1–Cdk4.

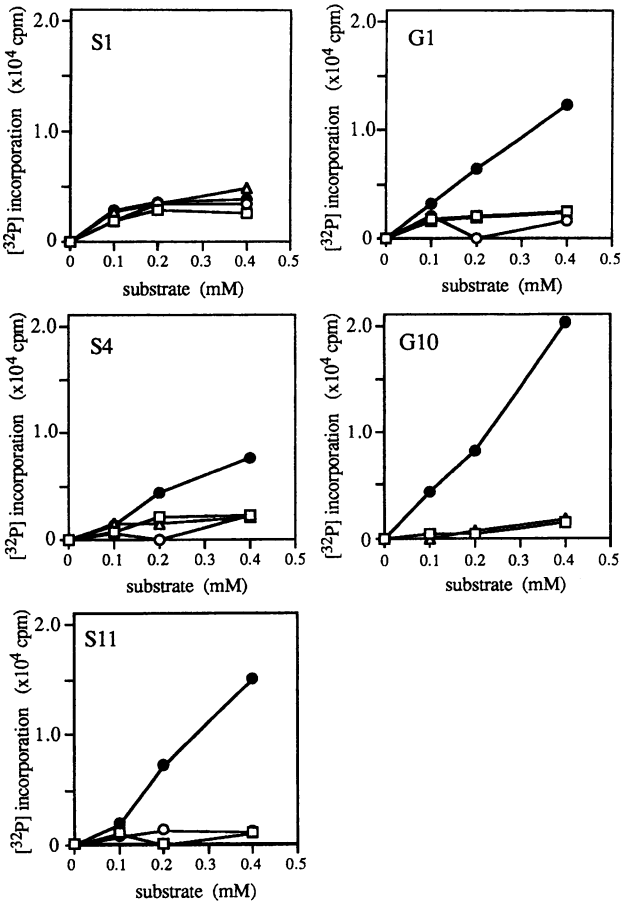

Fig. 2. Phosphorylation of synthetic peptides by lysates prepared from Sf9 cells transfected with cyclin D1- and/or Cdk4-virus. Whole-cell lysates were prepared from Sf9 cells infected with wild-type baculovirus (△) or with recombinant baculovirus that encoded cyclin D1 (○) or Cdk4 (□). A lysate containing cyclin D1–Cdk4 (●) was prepared from Sf9 cells co-infected with cyclin D1- and Cdk4-virus. Five μg of lysate were incubated with or without a synthetic peptide, such as G1, G10, S1, S4 and S11, in R buffer that contained [γ-^{32}P]ATP at 30°C for 30 min. The reaction mixture was then trapped on P81 paper, as described in Materials and methods. Incorporation of ^{32}P (the *y* axis) into the peptides was calculated from the difference in radioactivity between those with and those without each peptide in the reaction mixture.

Substrate specificities of other RB kinases

We confirmed the properties of cyclin D1–Cdk4 using native enzyme, precipitated with a specific antibody against Cdk4, from mammalian cells. Cdk2, Cdk4 and Cdk6 were immunoprecipitated from an extract of mouse FM3A cells, and each was specifically detected by Western blotting (Figure 3A). Furthermore, MAP kinases (such as ERK1 and ERK2) were detected in the cell lysates (Figure 3A, lanes 7 and 8) but they were found in neither the immunoprecipitates nor the preparations of purified cyclin–Cdks. pRB was phosphorylated by Cdk2, Cdk4 and Cdk6 that had been immunoprecipitated from FM3A cells (Figure 3B). In contrast, H1 histone was phosphorylated by Cdk2 but not by Cdk4 or Cdk6 (Figure 3B), as previously reported by Meyerson and Harlow (1994). The immunoprecipitated Cdk4 and Cdk6 phosphorylated S4, S11, S13, G1 and G11, whereas the S1 and S14 peptides were poor substrates for Cdk4 and Cdk6 (Figure 3C). Thus, our data demonstrated that Cdk6 had similar substrate

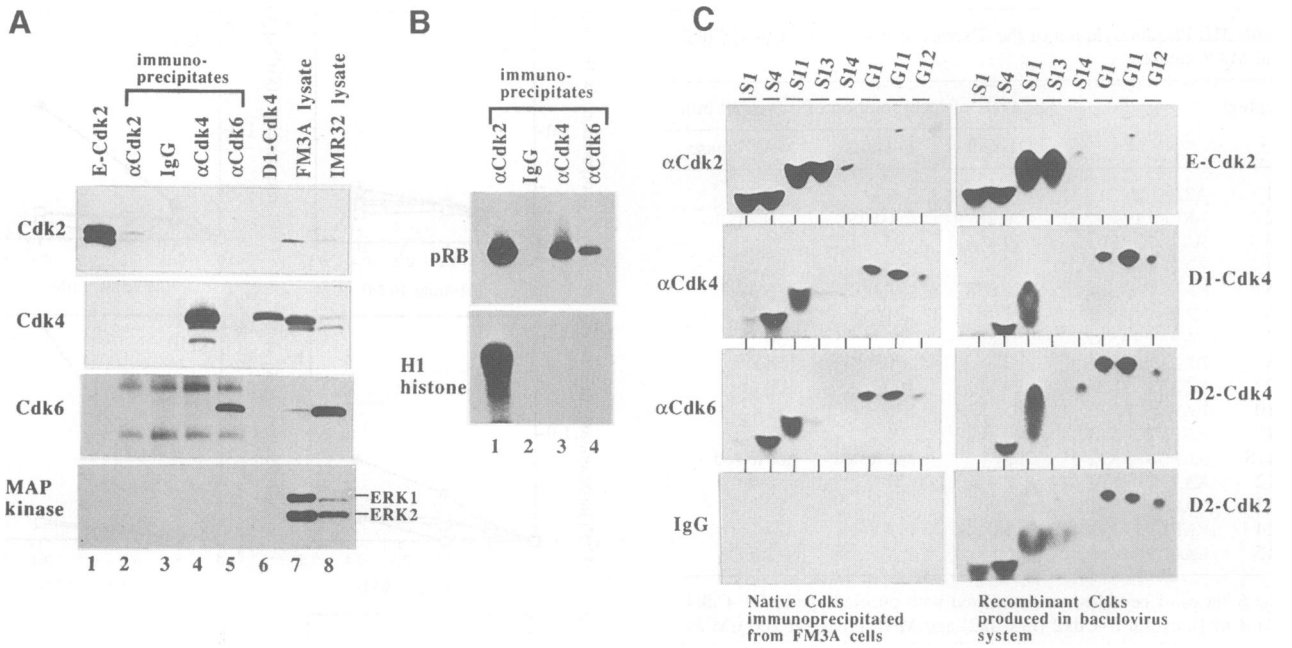

Fig. 3. Substrate specificities of RB kinases. Whole-cell lysates were prepared from mouse FM3A cells. The lysate was subjected to immunoprecipitation with antibodies against Cdk2 (αCdk2), Cdk4 (αCdk4) or Cdk6 (αCdk6) or control rabbit IgG (IgG), as described in Materials and methods. (**A**) The immunoprecipitates, purified cyclin E–Cdk2, cyclin D1–Cdk4, and lysates prepared from mouse FM3A and human IMR32 cells were subjected to Western blotting analysis with the antibodies against Cdk4, Cdk6, Cdk2 and MAP kinase. (**B**) The immunoprecipitates from FM3A cells were incubated with pRB or H1 histone in R buffer that contained [γ-^{32}P]ATP at 30°C for 30 min. Phosphorylated proteins were separated by SDS–PAGE (7.5% polyacrylamide) and detected by autoradiography. (**C**) Synthetic peptides such as S1, S4, S11, S13, S14, G1, G11 and G12 were incubated with immunoprecipitated kinase from FM3A cells or purified cyclin E–Cdk2 (E-Cdk2) or cyclin D1–Cdk4 (D1-Cdk4) or cyclin D2–Cdk4 (D2-Cdk4) or cyclin D2–Cdk2 (D2-Cdk2) in R buffer that contained [γ-^{32}P]ATP at 30°C for 30 min. The supernatants were then subjected to TLC on cellulose plates as described in Materials and methods. Spots visible in (C) represent phosphorylated peptides detected with the Bio-Image Analyzer (BAS2000).

specificity to Cdk4. It is confirmed that the substrate specificities of the recombinant enzymes were similar to the native Cdk4 and Cdk2 (Figure 3C). Moreover, we determined the substrate specificities of cyclin D2–Cdk4 and cyclin D2–Cdk2. Cyclin D2–Cdk4 efficiently phosphorylated S4, S11, S14, G1, G11 and G12 but scarcely S1 and S13. The tendency of the profile of phosphorylation by cyclin D2–Cdk4 resembled one by cyclin D1–Cdk4 with the exception of S14. On the contrary, cyclin D2–Cdk2 efficiently phosphorylated S1, S4, S11, S13, G1, G11 and G12. Thus, cyclin D2–Cdk2, which phosphorylates both pRB and H1 histone (Matsushime *et al.*, 1994), has intermediate specificity between cyclin E–Cdk2 and cyclin D2–Cdk4. These data suggest that recognition of the sequence surrounding the phosphorylation sites was influenced by both cyclin and Cdk.

Phosphorylation of Ser780 in pRB in vitro and in vivo

As described above, we showed that the G1 peptide containing the sequence present in pRB is selectively phosphorylated by cyclin D1–Cdk4 but not by cyclin E–Cdk2. Thus we asked whether Ser780 of RB is indeed phosphorylated by cyclin D1–Cdk4 *in vitro* and *in vivo*. To analyze the specific phosphorylation of Ser780 in pRB, we prepared specific antibody (αRB-P-Ser780) against phospho-Ser780 in pRB as described in Materials and methods. The results of ELISA showed that it selectively binds to the G1 peptide containing phospho-Ser780 but not to the unphosphorylated G1 peptide (data not shown). Selectivity of the antibody to phospho-Ser780 in pRB was tested using mutant GST–RB protein (GST–RB-S780L) in which Leu is substituted for Ser780. αRB-P-Ser780 recognized wild-type GST–RB phosphorylated by cyclin D1–Cdk4 but not wild-type GST–RB phosphorylated by cyclin E–Cdk2 (Figure 4, lanes 2 and 3). GST–RB-S780L, which had been phosphorylated by cyclin D1–Cdk4 as well as by cyclin E–Cdk2, was not recognized by αRB-P-Ser780 (Figure 4, lanes 5 and 6). These data strongly suggested that αRB-P-Ser780 can specifically detect the phosphorylation of Ser780 in pRB. We also tested phosphorylation by cyclin D2 complexes and found that cyclin D2–Cdk4 could also phosphorylate Ser780 in pRB (Figure 4, lane 13). Cyclin D2–Cdk2 could also weakly phosphorylate Ser780 in pRB (Figure 4, lane 16). These data were consistent with the data obtained from the S and G series of peptides as described above (Figure 3C). Ser780, in full-length pRB produced by baculovirus, was also phosphorylated by cyclin D1–Cdk4 but not cyclin E–Cdk2 (data not shown) .

Subsequently, we determined the phosphorylation of Ser780 in pRB *in vivo*. Serum-starved T98G cells were stimulated with 10% FCS and were harvested at 0, 2, 4, 6, 8, 10, 12, 14.5 and 17 h after serum stimulation. Cell-cycle analysis of T98G cells using flow-cytometry (Mayol *et al.*, 1995) and thymidine incorporation (data not shown) suggested that S phase began at 14 h after serum stimulation. As shown in Figure 5, cyclin D1 was expressed in G_1 phase from 4 to 12 h after serum stimulation. Cyclin E was constitutively expressed during the cell cycle in

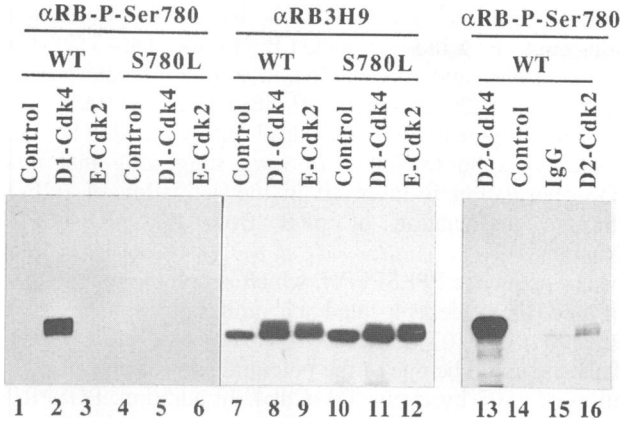

Fig. 4. Phosphorylation of Ser780 at the G1 site of pRB *in vitro*. The GST–RB-S780L in which Leu is substituted for Ser780 in wild-type GST–RB proteins was produced and purified as described in Materials and methods. The GST–RB-S780L (S780L) and the wild-type GST–RB (WT) proteins were incubated with or without (control) cyclin–Cdk in reaction buffer containing 100 μM cold ATP. These RB proteins were then subjected to SDS–PAGE (7.5% polyacrylamide) and were analyzed using Western blotting with anti-RB antibody 3H9 (αRB3H9) or the antibody against phospho-Ser780 in pRB (αRB-P-Ser780) as described in Materials and methods. Cyclin D1–Cdk4(D1-Cdk4), cyclin E–Cdk2 (E-Cdk2) and cyclin D2–Cdk4 (D2-Cdk4) were purified by HPLC. Sf9 lysate co-transfected with both Cdk2- and cyclin D2-viruses was immunoprecipitated by αCdk2 to prepare cyclin D2–Cdk2 (D2-Cdk2) or by IgG (IgG) for the control, as described in Materials and methods.

T98G cells as described previously by Mayol *et al.* (1995). Cdk4 expression gradually increased from 8 h after serum stimulation. The 33 kDa form of Cdk2, an active form, appeared beginning at 12 h after serum stimulation. To determine the expression of pRB, pRB was immunoprecipitated with αRB3H9 which can precipitate both phosphorylated- and underphosphorylated-pRB. The pRB was detected by Western blotting with αRB3H9. Then the membrane was reprobed with αRB-P-Ser780 (Figure 5). It was clear that Ser780 in pRB was phosphorylated in T98G cells. Furthermore, the phosphorylation of Ser780 in pRB began at 8 h after serum stimulation and continued at least until early S phase (14.5–17 h). The timing of the phosphorylation of Ser780 in pRB coincides with the appearance of hyper-phosphorylated pRB which migrates slowly in the sample of αRB3H9. Phosphorylation of Ser780 was detected in the hyperphosphorylated form of pRB (hy-p-RB) but not in the underphosphorylated form (un-p-RB). Therefore, Ser780 in pRB is indeed phosphorylated *in vivo* in a cell cycle-dependent manner and the start of phosphorylation of Ser780 was correlated with that of hyperphosphorylation of pRB.

It is well known that pRB binds with E2F-1 to suppress its activity. We investigated the correlation between the phosphorylation of Ser780 in pRB and pRB-E2F-1 complex formation to study the physiological significance of the phosphorylation of Ser780 in pRB (Figure 6). For this purpose, whole cell lysates prepared from WI38 cells were subjected to immunoprecipitation by αRB-P-Ser780 or αRB3H9. Immunoprecipitates were analyzed by Western blotting with αE2F-1, αRB-P-Ser780 and αRB3H9. E2F-1 was not detected in the immunoprecipitate of pRB phosphorylated at Ser780 (lane 2), whereas E2F-1 was co-precipitated with pRB precipitated by

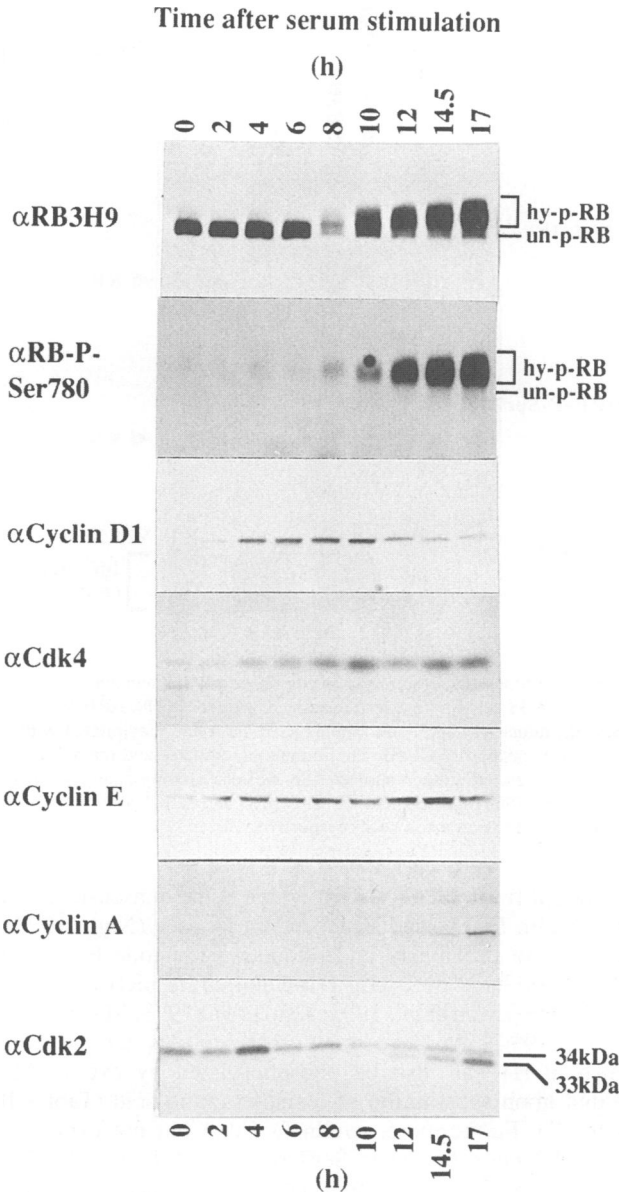

Fig. 5. Cell-cycle dependent phosphorylation of Ser780 in pRB. Serum-starved T98G cells were stimulated with fresh DMEM medium containing 10% FCS. The cells were harvested at the indicated times (0, 2, 4, 6, 8, 10, 12, 14.5 and 17 h) after serum starvation as described in Materials and methods. Immunoprecipitated pRB from the whole-cell lysates (250 μg protein) with αRB3H9 was subjected to Western blotting analysis using αRB3H9 and αRB-P-Ser780. Hyper- and under-phosphorylated forms of pRB were indicated as hy-p-RB and un-p-RB, respectively. The lysates (40 μg protein) were subjected to Western blotting with antibodies such as αCyclin D1, αCyclin E, αCyclin A, αCdk2 and αCdk4. Active and inactive forms of Cdk2 were indicated as 33 kDa and 34 kDa respectively.

αRB3H9 (lane 3). In converse experiments in the immunoprecipitates by αE2F1, pRB was detected by αRB3H9 but not by αRB-P-Ser780 (Figure 6, lane 7), suggesting that pRB cannot bind E2F-1 when Ser780 is phosphorylated.

Discussion

We demonstrate here that the consensus amino acid sequence for phosphorylation by cyclin D1–Cdk4 is

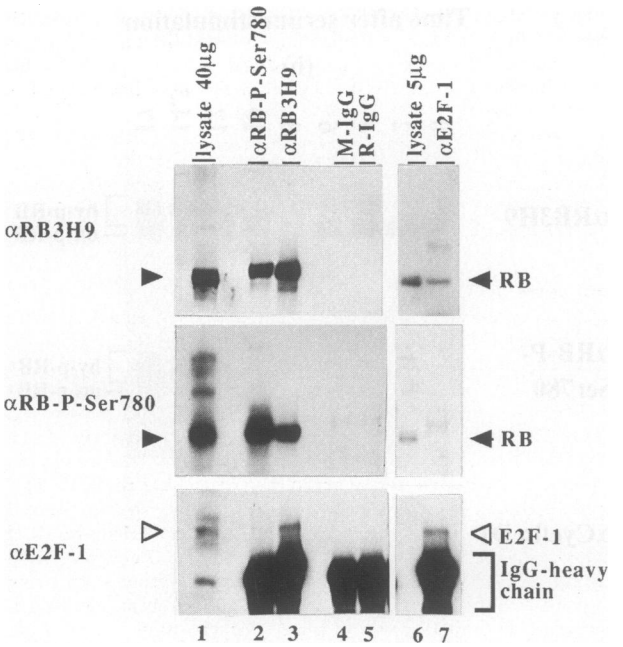

Fig. 6. Absence of phosphorylated Ser780 in pRB associated with E2F-1. WI38 cell lysates were incubated with αRB3H9, αRB-P-Ser780, mouse-(M-IgG) and rabbit-IgG(R-IgG) and precipitated with protein A–Sepharose CL4B. The immunoprecipitates and the WI38 lysates (lysate) were then subjected to Western blotting analysis using αRB-P-Ser780, αRB3H9 and αE2F-1. pRB and E2F-1 are indicated as closed and open arrowheads respectively.

different from S/T-P-X-K/R, which is the consensus motif for cyclin E-, A- and B-dependent kinases (Nigg, 1993). It was not previously understood why histone H1 is not phosphorylated by cyclin D1–Cdk4 while pRB is a good substrate (Ewen *et al.*, 1993; Kato *et al.*, 1993; Matsushime *et al.*, 1994). We have shown that peptides derived from histone H1 can also be phosphorylated by cyclin D1–Cdk4 upon substitution of a single amino acid (Tables II and III). Furthermore, cyclin D–Cdk4 but not cyclin A/E–Cdk2 phosphorylates Ser780 in the G1 site of pRB. Ser780 in pRB is phosphorylated in G_1 phase during the cell cycle. Cyclin Ds–Cdk4 will gain their accessibilities to pRB through direct interaction of cyclin Ds with pRB (Kato *et al.*, 1993) and recognize localized sequences around the phosphorylation sites in pRB to phosphorylate the specific site(s), such as Ser780.

The only confirmed target for cyclin D1–Cdk4 is pRB (Sherr, 1995), although Myo D has also been suggested to be a target (Skapek *et al.*, 1995). However, several other cyclin–Cdks, including cyclin E–Cdk2, seem to be involved in the phosphorylation of pRB *in vivo* (Weinberg, 1995), and more than 10 sites in pRB are phosphorylated (Lees *et al.*, 1991; Mittnacht *et al.*, 1994). Because this cell cycle-dependent phosphorylation of pRB plays a key role in both the control of cellular proliferation and carcinogenesis (Helin and Harlow, 1993; Weinberg, 1995), it is important to understand the functional difference of these cyclin–Cdks and phosphorylation sites. Our data suggested that at least one site in pRB, Ser780, can be phosphorylated *in vivo* by cyclin D–Cdk4 but not by cyclin A/E–Cdk2, while several other sites are phosphorylated by both cyclin D–Cdk4 and cyclin A/E–Cdk2 and some others are specific to cyclin A/E/B–Cdk. The cyclin D–Cdk4-specific residue Ser780 is located in a position adjacent to E1A binding pocket B (residues 646–772) (Hu *et al.*, 1990) and is included in a region required for binding to E2F (residues 393–869, Hiebert, 1993). As shown in Figure 6, pRB that was phosphorylated in Ser780 could not bind to E2F-1 *in vivo*, suggesting that this phosphorylation is involved in the activation of E2F-1 through dissociation of pRB from the pRB-E2F-1 complex.

The sequence PPLSPFPH, which is homologous to that of the G1 peptide, is located at a similar position (residues 961–969) of p107 (Ewen *et al.*, 1991; Zhu *et al.*, 1993). This site could be one of the potential sites of phosphorylation of p107 by cyclin D1–Cdk4. In addition, PGSPRR in p107 is a common motif for phosphorylation by both Cdk4 and Cdk2. As p107 contains four consensus sequences for phosphorylation by Cdk2, namely EIGTPRK, IYISPHK, FNGSPSK and DAESPAK, Cdk2 may have the potential to phosphorylate p107. Therefore, p107 could be phosphorylated not only by cyclin D1–Cdk4 but also by cyclin A–Cdk2 *in vitro*. However, Beijerbergen *et al.* (1995) have reported that phosphorylation of p107 is regulated by cyclin D1–Cdk4 but not by cyclin A/E–Cdk2 *in vivo*, using transfection experiments. The discrepancy between *in vivo* and *in vitro* results is still unclear.

The consensus motif for cyclin D1–Cdk4 identified here resembles that of MAP kinase (Gonzalez *et al.*, 1991). However, MAP kinase is distinct from cyclin D1–Cdk4 in its dependence on the phase of the cell cycle and its mechanism of activation (Muller *et al.*, 1993).

The G_1/S transition in mammalian cells appears to be regulated by D-type cyclins, associated with either Cdk4 or Cdk6 (Bates *et al.*, 1994; Meyerson and Harlow, 1994), and by cyclin E in association with Cdk2 (Sherr and Roberts, 1995). This notion is supported by several lines of evidence. (i) D- and E-type cyclins are expressed and form active kinase complexes at middle and late G_1 phase (Sherr, 1995). (ii) Microinjection of cyclin D1 antisense plasmid or monoclonal antibodies against cyclin D1 prevents entry into S phase (Baldin *et al.*, 1993; Quelle *et al.*, 1993). Likewise, entry into S phase is blocked by inhibition of Cdk2, the functional partner of cyclin E, either by microinjection of antibodies (Pagano *et al.*, 1993; Tsai *et al.*, 1993) or transfection with a dominant negative Cdk2 mutant (van den Heuvel and Harlow, 1993). (iii) Overexpression of cyclin D and E, but not of cyclin B1, shortens the length of G_1 phase in various mammalian cells (Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994; Wimmel *et al.*, 1994). In collaboration with cyclin D-dependent kinases, cyclin E–Cdk2 may contribute to the phosphorylation of pRB late in G_1 phase since ectopic expression of cyclin E in human osteosarcoma cells causes phosphorylation of pRB (Hinds *et al.*, 1992).

Cyclins E and D1 seem, however, to control two different events during the G_1/S transition because cyclin E, but not D1, is essential for entry into S phase in mammalian cells that lack a functional pRB (Lukas *et al.*, 1995a; Ohtsubo *et al.*, 1995). Moreover, introduction of cyclin D1 triggers the rapid phosphorylation of pRB but that of cyclin E does not (Resnitzky and Reed, 1995). It has been suggested that pRB might represent the sole

critical substrate of D-type Cdks (Sherr and Roberts, 1995). Consistent with this hypothesis, overexpression of p16 (a Cdk4- and Cdk6-specific inhibitor) prevents proliferation in pRB-positive cells but is ineffective in pRB-negative cells (Guan *et al.*, 1994; Lukas *et al.*, 1995b; Medema *et al.*, 1995). Thus, it is suggested that cyclin E–Cdk2 phosphorylates other key substrates in addition to pRB, perhaps acting to trigger the actual onset of DNA replication once cells have passed the R point (Sherr and Roberts, 1995). We demonstrated that the consensus motif for phosphorylation by cyclin D1–Cdk4 is different from that by cyclin A/E–Cdk2. Our findings strongly suggest the possibility that Cdk2 and Cdk4 could each have a specific target. It may be possible to identify potential substrates for cyclin E–Cdk2 or cyclin D–Cdk4 from a search for the consensus amino acid sequences for phosphorylation by these Cdks in databases. We searched the consensus motif for phosphorylation by cyclin D1–Cdk4 in GenBank and Swiss Prot. As expected, RB and p107 were hit in the databases. We also found two viral proteins, as polyomavirus large T antigen (PKTPPPK) and human papilloma virus-57 E2 protein (PSSPPPK) which are involved in carcinogenesis and abrogation of the cell cycle in the host cells. SV40 large T antigen has a low-potency sequence (FFLTPHR) for Cdk4. Furthermore, the catalytic subunit of the protein phosphatase type 1, PP-1α2, has the motif for phosphorylation by Cdk4 (PITPPR). Interestingly, PP-1α2 and large T antigens associate with the same region of pRB (Durfee *et al.*, 1993). It has been suggested that PP-1α2 regulates dephosphorylation of pRB from mitosis to early G_1 during the cell cycle. Therefore, D-type cyclin–Cdk4 may inhibit the activity of PP-1α2 or complex formation of pRB with PP-1α2 in mid–late G_1 phase through phosphorylation.

The difference between the fundamental roles of cyclins D and E is unclear at present. Our new findings provide a fresh clue that should help in future studies of the function of these cyclins.

Materials and methods

Cell culture

Mouse mammary tumor cell line FM3A was cultured in RPMI1640 medium containing 10% fetal bovine serum. Human fibroblast WI38 and human glioblastoma T98G were grown at 37°C on 75 cm^2 tissue culture flask in DMEM medium containing 10% fetal calf serum (FCS). T98G cells were subjected to serum-starvation in MCDB-105 medium without FCS for 3 days, then stimulated with fresh DMEM medium containing 10% FCS as previously described by Mayol *et al.* (1995).

Preparation of proteins and synthetic peptides for use as substrates

Human E2F-1 cDNA was cloned into the pGEX2T vector (Pharmacia, Uppsala, Sweden) and glutathione S-transferase fused E2F-1 protein (GST–E2F-1) was produced in *Escherichia coli* cells that carried this plasmid by incubation with 0.1 mM isopropyl-β-D(−)-thiogalactopyranoside (IPTG) at 25°C for 12 h. GST–E2F-1 protein was adsorbed to glutathione–Sepharose CL4B (Pharmacia) and eluted with 10 mM reduced glutathione (Kitagawa *et al.*, 1995). After dialysis against phosphate-buffered saline, the affinity purified GST–E2F-1 was concentrated to 0.3 mg/ml. pRB was purified from Sf9 cells that had been infected with the recombinant virus encoding full-length cDNA for human pRB by sequential chromatographies on phosphocellulose, heparin–Sepharose and Q–Sepharose (Kitagawa *et al.*, 1994). Human p53 was also produced by the baculovirus system. Human H1 histone was obtained from Boehringer Mannheim Co. Ltd (Mannheim, Germany). Sea-star MAP kinase ($p44^{mpk}$; erk1) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Substrate peptides, such as the RB series (see Table I), the S series (see Tables II and III) and the G series (see Tables II and IV), were synthesized and purified by HPLC at the Peptide Institute Co. Ltd (Osaka, Japan) or Sawaday Technology Co. Ltd (Tokyo, Japan).

Leu was substituted for Ser780 in wild-type GST–RB (pGEXRB; Saijo *et al.*, 1994) using site-directed mutagenesis kit (Promega). The wild-type GST–RB and GST–RB-S780L proteins were produced in *E.coli* treated with 1 mM IPTG, and purified using glutathione–Sepharose CL-4B (Pharmacia) as described by Saijo *et al.* (1994).

Antibodies

Cyclin D1-specific and cyclin E-specific polyclonal antibodies were raised against C-terminal peptides of human cyclin D1 and cyclin E, respectively, in collaboration with MBL Co. Ltd (Ina, Japan). Anti-Cdk4 (C-22), anti-Cdk6 (C-21) and anti-Cdk2 (M2) antibodies, raised against the C-terminal sequence of human Cdk4, Cdk6 and Cdk2 respectively, were obtained commercially from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The MAP kinase-specific antibody (K-23) and cyclin A-specific antibody were also obtained from Santa Cruz Biotechnology, Inc. and Upstate Biotechnology Inc. respectively. The monoclonal antibody against p107 (SD7) was a gift from Dr E.Harlow (MGH Cancer Center, Boston, MA, USA). Anti-RB-phospho-Ser780 polyclonal antibody (αRB-P-Ser780) was raised against G1 peptide containing phospho-Ser780, RPPTLS(PO_3)PIPHIPR, which was chemically synthesized using an arylthio group to protect the phosphate (Ueno *et al.*, 1995). Anti-serum obtained from an immunized rabbit was purified using column chromatography of Sepharose CL-4B conjugated with phospho-G1-peptide. The affinity purified αRB-P-Ser780 was then passed through column chromatography of Sepharose CL-4B conjugated with G1 peptide to deplete contaminated antibodies which can bind with unphosphorylated antigen.

Production and purification of cyclin-dependent kinases

Human cDNAs for cyclin A, cyclin D1, cyclin E, Cdk2 and Cdk4 were cloned into the baculovirus expression vector pVL1393 (PHARMINGEN, San Diego, CA, USA) as previously described (Kitagawa *et al.*, 1995). Active cyclin–Cdk complexes were produced in Sf9 cells that had been co-infected with recombinant baculoviruses encoding cDNAs for cyclin (A, D1 or E) and Cdk (2 or 4) (Higashi *et al.*, 1996). Whole-cell lysates were prepared by sonication of the cells in IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20 and 10% glycerol) that contained protease inhibitors [1 mM PMSF, 5 μg/ml each of nafamostat mesilate (Banyu Pharmaceutical Co. Ltd, Tokyo, Japan), antipain, pepstatin, leupeptin, chymostatin and E-64 (Peptide Institute Co. Ltd, Osaka, Japan)], and centrifuged at 15 000 r.p.m. (18 600 *g*) for 15 min. Complexes of mouse cyclin D2 with human Cdk2 and human Cdk4 were also prepared by the same method. Proteins were purified by column chromatographies on Mono Q (Pharmacia), hydroxyapatite and Mono S (Pharmacia). Cyclin D1 and Cdk4 protein were traced by Western blotting with antibodies against cyclin D1 and Cdk4 respectively, and by *in vitro* kinase assay with purified pRB and H1 histone as substrates during purification. The proteins formed an active complex.

In vitro kinase assay

In vitro kinase assays using purified proteins and purified enzymes were performed as previously reported (Kitagawa *et al.*, 1993, 1995). Substrate proteins were incubated with purified cyclin A–Cdk2, cyclin E–Cdk2 or cyclin D1–Cdk4 at 30°C for 30 min in R buffer (20 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 4.5 mM 2-mercaptoethanol, 1 mM EGTA) that contained 50 μM ATP and 10 μCi [γ-^{32}P]ATP (6000 Ci/mmol; Amersham, Buckinghamshire, UK) in a final volume of 25 μl. Phosphorylated proteins were analyzed by SDS–PAGE (7.5% polyacrylamide) with subsequent autoradiography.

In vitro kinase assays using synthetic peptides and purified enzymes were performed as previously reported (Taya *et al.*, 1989; Kitagawa *et al.*, 1992; Higashi *et al.*, 1995). Synthetic peptides, such as the RB series, S series and G series, were incubated with purified cyclin A–Cdk2, cyclin E–Cdk2, cyclin D1–Cdk4 or MAP kinase at 30°C for 30 min in R buffer that contained 50 μM ATP and 0.6 μCi [γ-^{32}P]ATP in a final volume of 25 μl. Assays were terminated by adding 10 μl of 350 mM H_3PO_4. Peptides were trapped on P81 papers (Whatman Co. Ltd, Maidstone, UK) which were washed six times with 75 mM H_3PO_4 and then monitored for radioactivity in a liquid scintillation counter.

In vitro kinase assays using immunoprecipitated Cdks from mammalian cells

Whole cell lysates were prepared from mouse FM3A cells in IP buffer. Lysates (4 mg protein /ml) were treated with protein A–Sepharose CL-4B (Sigma, St Louis, MO, USA) to avoid non-specific binding and were centrifuged. The supernatants were incubated with antibodies against Cdk2, Cdk4 or Cdk6, or control rabbit IgG and then with protein A–Sepharose CL-4B. After centrifugation, the immunoprecipitates were washed five times with IP buffer. The immunopurified Cdks and substrates were incubated at 30°C for 30 min in R buffer that contained 50 μM ATP and 4 μCi [γ-^{32}P]ATP in a final volume of 25 μl. The supernatants were separated by TLC on cellulose plates with solvent A (*n*-butanol-ethanol-25% ammonia water-chloroform, 4:5:9:2, by vol) as a mobile phase (Kitagawa *et al.*, 1992). Phosphorylated peptides were detected with a Bio-Image Analyzer (BAS2000, Fuji, Tokyo, Japan).

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